Lithium interactions with Na\(^+\)-coupled inorganic phosphate cotransporters: insights into the mechanism of sequential cation binding

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Submitted 30 September 2011; accepted in final form 8 November 2011

Lithium interactions with Na\(^+\)-coupled inorganic phosphate cotransporters: insights into the mechanism of sequential cation binding. Am J Physiol Cell Physiol 302: C539–C554, 2012. First published November 9, 2011; doi:10.1152/ajpcell.00364.2011.—Type Ia/b Na\(^+\)-coupled inorganic phosphate cotransporters (NaPi-IIa/b) are considered to be exclusively Na\(^+\) dependent. Here we show that Li\(^+\) can substitute for Na\(^+\) as a driving cation. We expressed NaPi-IIa/b in *Xenopus laevis* oocytes and performed two-electrode voltage-clamp electrophysiology and uptake assays to investigate the effect of external Li\(^+\) on their kinetics. Replacement of 50% external Na\(^+\) with Li\(^+\) reduced the maximum transport rate and the rate-limiting plateau of the Pi-induced current began at less hyperpolarizing potentials. Simultaneous electrophysiology and \(2^{32}\)Na uptake on single oocytes revealed that Li\(^+\) ions can substitute for at least one of the three Na\(^+\) ions necessary for cotransport. Presteady-state assays indicated that Li\(^+\) ions alone interact with the empty carrier; however, the total charge displaced was 70% of that with Na\(^+\) alone, or when 50% of the Na\(^+\) was replaced by Li\(^+\). If Na\(^+\) and Li\(^+\) were both present, the midpoint potential of the steady-state charge distribution was shifted towards depolarizing potentials. The charge movement in the presence of Li\(^+\) alone reflected the interaction of one Li\(^+\) ion, in contrast to 2 Na\(^+\) ions when only Na was present. We propose an ordered binding scheme for cotransport in which Li\(^+\) competes with Na\(^+\) to occupy the putative first cation interaction site, followed by the cooperative binding of one Na\(^+\) ion, one divalent P\(_i\) anion, and a third Na\(^+\) ion to complete the carrier loading. With Li\(^+\) bound, the kinetics of subsequent partial reactions were significantly altered. Kinetic simulations of this scheme support our experimental data.

Electrophysiology: *Xenopus* oocyte; presteady-state; stoichiometry

SECONDARY-ACTIVE COTRANSPORTERS use the free energy available from downhill cotransport of specific solutes to catalyze uphill transport of other specific solutes. The majority of mammalian cotransporters use Na\(^+\) as their preferred driving cation under physiological conditions, yet in some cases other cations (Li\(^+\), H\(^+\)) can substitute for Na\(^+\), albeit with altered apparent cation and substrate affinities and transport rates. For example, Li\(^+\) or H\(^+\) can drive glucose transport catalyzed by the sodium-coupled glucose cotransporter (SGLT1; Refs. 17, 18) and Li\(^+\) can substitute for Na\(^+\) as the driving cation of the sodium-coupled dicarboxylate transporter (NaDC-1; Ref. 24) and the excitatory amino acid transporter (EAAT1; Ref. 3). Such cation substitutions can have significant clinical consequences. For example, therapeutic doses of orally administered LiCl, used to treat bipolar disorder, result in the rapid onset of increased renal excretion of Krebs cycle intermediates (31). Typically, the concentration of Li\(^+\) ([Li\(^+\)]) in both serum and glomerular filtrate is in the millimolar range. Although this is ≥10-fold smaller than the prevailing Na\(^+\) concentration ([Na\(^+\)]), it is sufficient to compete with Na\(^+\) for one of the three cotransport sites postulated for NaDC-1 (24) and thereby inhibit dicarboxylate reabsorption. On the other hand, some cotransporters show no detectable transport activity if Li\(^+\) fully replaces Na\(^+\), yet when both cations are available, potent stimulation of transport can occur at membrane potentials in the physiological range, as reported for the γ-amino acid transporter GAT1 (19, 20, 33).

Type II Na\(^+\)-coupled inorganic phosphate (Pi) cotransporters (NaPi-IIa,c; SLC34A1,3) are both expressed in the apical membrane of mammalian proximal tubule epithelia where they mediate P\(_i\) reabsorption (8, 23). Although genetically unrelated to NaDC-1, their 3:1 (for electrogenic NaPi-IIa) and 2:1 (for electroneutral NaPi-IIc) Na\(^+\):Pi stoichiometries imply the existence of multiple Na\(^+\) binding sites that could also be potential targets for Li\(^+\) interaction in the kidney. In one of the few reports (25) that has addressed the effects of Li\(^+\) on renal P\(_i\) handling, it appears that P\(_i\) clearance and tubular reabsorption are normal in humans undergoing chronic Li\(^+\) therapy, unlike the case for dicarboxylates. Moreover, in two animal studies, the effect of high doses of Li\(^+\) on P\(_i\) handling would appear to result from an indirect action on PTH regulation of NaPi-II abundance in the brush border membrane, as opposed to an interaction with cation binding sites within the transport protein (2, 32).

In contrast to the lack of any clinically significant effect of Li\(^+\) on renal P\(_i\) transport, biophysical studies on heterologously expressed NaPi-II proteins in *Xenopus* oocytes reveal direct interactions of Li\(^+\) with the protein (15, 29, 30). By labeling the electronegative isomorph flounder NaPi-IIb with a fluorophore at functionally important sites, voltage-dependent changes in emitted fluorescence intensity that depend on Li\(^+\) activity offer compelling evidence that before Pi binding one Li\(^+\) ion interacts with the protein. The putative conformational changes, as reported by fluorescence intensity changes, were comparable to those reported for Na\(^+\) over the same concentration range (29); however, in these studies, no evidence for the cotransport of Li\(^+\) was presented. Similar Li\(^+\)-dependent changes in fluorescence emissions were reported for the electroneutral NaPi-IIc (15). It was also significant that in that study no change in \(32^{\text{P}}\) uptake was observed when 50% of external Na\(^+\) was replaced with Li\(^+\), which suggests that Li\(^+\) does not readily substitute for Na\(^+\) in the complete cotransport cycle of the electroneutral NaPi-Iic.

Taken together, these findings prompted us to undertake a rigorous investigation of the effects of Li\(^+\) on NaPi-II transport kinetics using electronegative NaPi-II isoforms expressed in *Xenopus laevis* oocytes. Here we present evidence that 1) two Na\(^+\) ions interact sequentially with the carrier before P\(_i\) bind-
ing and 2) \(\text{Li}^+\) ions can compete with \(\text{Na}^+\) ions at the cation binding site that is first occupied. Moreover, we demonstrate that at sufficiently high concentrations one \(\text{Li}^+\) can substitute for one \(\text{Na}^+\) to drive cotransport of \(\text{P}_i\). Our findings offer valuable mechanistic insight into how different cations can interact with cation-driven carrier proteins to drive coupled transport.

**MATERIALS AND METHODS**

**Solutions and Reagents**

Three standard extracellular solutions were used, each containing the following (in mM): 2 KCl, 1.8 CaCl\(_2\), 0.82 MgCl\(_2\), and 10 HEPES-Tris, adjusted to pH 7.4 with either 100 NaCl, 100 LiCl, or 100 choline Cl, and are referred to as 100Na, 100Li, or 100Ch solutions, respectively. In cation substitution experiments NaCl was equimolarily replaced with either ChCl or LiCl and solutions with intermediate extracellular concentrations of Na and Li were obtained by mixing 100Na with 100Ch or 100Li in appropriate proportions to maintain the same ionic strength. \(\text{P}_i\) was added from a 1 M \(\text{KH}_2\text{PO}_4/\text{K}_{2}\text{HPO}_4\) stock premixed to give pH 7.4. Modified Barth’s solution for storing the following (in mM): 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), and 10 HEPES-MATERIALS AND METHODS for one Na

**Bessel characteristic). Faster sampling rates (up to 20 k samples/s)** were used for voltage-step recordings with low-pass filtering adjusted accordingly (typically at 500 Hz).

Steady-state \(\text{P}_i\) activation of electrotransporters was determined by varying the \(\text{P}_i\) concentration ([\(\text{P}_i\)]) in the presence of the same background solution and subtracting the respective currents in the control solution alone from those in control solution + \(\text{P}_i\). Steady-state \(\text{Na}^+\) activation was similarly determined by subtracting the respective responses in the control solution from those in control + \(\text{P}_i\) and [\(\text{P}_i\)] = 1 mM.

Steady-state \(\text{P}_i\)-induced currents (\(I_{\text{P}_i}\)) were fit with a form of the modified Hill equation:

\[
I_{\text{P}_i} = I_{\text{P}_i}^\text{max} \left( \frac{[S]^{n_H}/([S]^{n_H} + (K_{0.5})^{n_H})} + K \right)
\]

where [\(S\)] is the concentration of variable substrate (\(\text{Na}^+, \text{Li}^+, \text{or P}_i\), \(I_{\text{P}_i}^\text{max}\) is the maximal \(\text{P}_i\)-induced current, \(K_{0.5}\) is the apparent affinity constant for substrate \(S\), \(n_H\) is the Hill coefficient, and \(K\) is a constant that takes in account of uncoupled leak effects (4). For \(\text{P}_i\) activation, \(n_H = 1\) and Eq. 1 reduces to a Michaelian form.

Presteady-state relaxations were recorded using voltage steps from a holding voltage, \(V_h\), of \(-60\) mV to voltages in the range of \(-180\) to \(+80\) mV. Unless otherwise stated, relaxations were quantified by fitting with a two-component exponential function starting \(~4\) ms after the commencement of the voltage step to allow the linear components of charge movement arising from charging the oocyte capacitance to completely decay. The fitted curve was then extrapolated back to the time at which 70% of the membrane was charged (typically \(1\) ms after pulse onset), estimated by integrating the capacity transient for a 20-mV step from the holding potential. This time point was used as the reference time for numerical integration of the total relaxation to obtain the charge moved (\(Q\)) for a step from the holding potential to the test potential.

The \(Q-V\) data were fitted with a Boltzmann function of the form:

\[
Q = Q_{\text{hyp}} + Q_{\text{max}}/\left[1 + \exp\left[ze(V_{0.5} - V)/kT\right]\right]
\]

where \(Q_{\text{hyp}}\) is the voltage at which the charge is distributed equally between two hypothetical states, \(z\) is the apparent valency of an equivalent charge that moves through the whole of the membrane field, \(Q_{\text{max}}\) is the total charge available to move, \(Q_{\text{hyp}}\) is the charge of the hyperpolarizing limit and is a function of \(V_h\), and \(e, k, T\) have their usual meanings.

**Radiolabeled Tracer Experiments**

\(^{32}\text{P}\) uptake assay. Oocytes expressing \(\text{flNaPi-IIb}\) and noninjected oocytes (6–10 oocytes/group) were first equilibrating in 100Na solution without tracer. After aspiration of this solution, oocytes were incubated in 100Na solution containing 1 mM cold P, and \(^{32}\text{P}\) (final specific activity of 10 mCi/mmol; Perkin Elmer). Uptake proceeded for 10 min and then oocytes were washed three times with 5 ml of ice-cold 100Ch solution containing 2 mM P, and lysed individually in 250 \(\mu\)l of 4% SDS. The amount of radioactivity in each oocyte was measured by scintillation counting (Tri-Carb 2900TR; Packard). The uptake time was chosen to be short enough to assume that initial rate conditions were satisfied so that uptake/unit time is a direct measure of transport velocity, without compromising measurement reliability, based on previous studies (21). The data were normalized for the uptake obtained from oocytes expressing \(\text{flNaPi-IIb}\) WT in standard condition (100Na or 50Na50Ch) as indicated.

Simultaneous voltage-clamp and \(^{22}\text{Na}\) uptake. The standard TEVC was used as described previously (28). The oocyte was impaled and clamped at a constant voltage and then superfused with a solution \(40\text{Na60Ch}\) or \(40\text{Na60Li}\) until a stable base line was reached. The oocyte was exposed to the corresponding solution containing 1 mM P, cold and \(^{22}\text{Na}\) (final specific activity 1.5 mCi/mmol; Perkin Elmer, Switzerland) for 10 min. After the baseline returned to the starting level, the oocyte was removed from the chamber and washed three times in ice-cold 100Ch

**Expression in Xenopus Laevis Oocytes**

Female \(X.\) \(\text{laevis}\) frogs were purchased from \(Xenopus\) Express. Portions of ovaries were surgically removed from frogs anesthetized in MS222 (tricaine methanesulphonate) and cut in small pieces. After the incision was sutured, the frog was placed in a separate tank for \(\geq2\) days to fully recover from the surgery and returned to a larger tank. A minimum of 8 wk were allowed before operation on the same animal. All procedures for animal handling and harvesting \(X.\) \(\text{laevis}\) oocytes were approved in writing by the University of Zurich and the Swiss Federal Veterinary Authorities.

Oocytes were typically treated for 45 min with collagenase (crude type 1A) 1 mg/ml in 100Na solution (without Ca\(^{2+}\)) in presence of 0.1 mg/ml trypsin inhibitor type III-O. Healthy stage V-VI oocytes were selected, maintained in modified Barth’s solution at 16°C, and injected with 10 ng of cRNA. Experiments were performed 4–7 days after injection.

**Two-Electrode Voltage Clamp**

The standard two-electrode voltage clamp (TEVC) technique was applied using either Geneclamp (Molecular Devices), Turbo TEIX0 (NPI Electronics; Tamm, Germany), or a laboratory-built TEVC instrument (5). Oocytes were mounted in a small recording chamber (volume: 100 \(\mu\)l) and continuously superfused (5 ml/min) with the test solution precoulooled to 20°C. For constant voltage recordings, currents were acquired at 20 samples/s and filtered at 10 Hz (3 dB, 8-pole Bessel characteristic). Faster sampling rates (up to 20 k samples/s) were used for voltage-step recordings with low-pass filtering adjusted accordingly (typically at 500 Hz).
solution. This experiment was repeated using a solution containing 20 mM Na\(^+\) and 80 mM Li\(^+\) or 80 mM choline Cl.

The oocyte was lysed as described above. The net charge (Q) was calculated from the current traces by subtracting the endogenous holding current and numerically integrating the area under the baseline-corrected curve. Uptake and charge were expressed as their molar equivalents by assuming monovalency for the charge.

**Data Analysis and Software**

Data analysis was performed with Clampfit V. 10.2 (Molecular Devices) and Prism V. 4.03 (Graphpad Software, La Jolla, CA). Data points are shown as means ± SE. Simulations were performed using Berkeley Madonna V8.0.2a8 software (www.berkeleymadonna.com).

**RESULTS**

**Effect of Li\(^+\) Substitution on 32P Uptake in Oocytes Expressing Rat and Flounder Isoforms**

For oocytes expressing the flNaPi-IIb isoforms, replacement of 100 mM NaCl with 100 mM LiCl (100Li) in the incubation medium gave a small uptake amounting to 3.0 ± 0.1% of that measured in 100Na (Fig. 1A, top). Similar behavior was observed in oocytes that expressed the rat NaPi-IIa isoform (data not shown). Noninjected (NI) oocytes from the same donor animals showed no significant difference between Li\(^+\) and Na\(^+\) incubation, which suggested that the small uptake using 100Li for the NaPi-IIa/b expressing oocytes was not an endogenous artifact (Fig. 1A, bottom). In a second series of uptake experiments, we replaced 50% of external NaCl with either LiCl (50Na50Li) or choline Cl (50Na50Ch) and compared the uptake for each isoform. The uptake in the presence of Li\(^+\) was reduced by ~50% (Fig. 1B). This suggested an interaction of Li\(^+\) with the transporter. The effect of Li\(^+\) appeared to be common to all electrogenic isoforms, as we obtained similar results using the human NaPi-IIa (data not shown).

**Steady-State Electrophysiology**

To gain further insight into the underlying mechanism of Li\(^+\) interactions, we used the TEVC to define the electrical driving force and measure the \(I_P\) under different external perfusion conditions, in response to 1 mM Pi (pH 7.4; Fig. 2). All these and subsequent assays were performed on oocytes expressing the flounder NaPi-IIb isoform because it gave a more robust expression. We also performed control measurements using either the rat or human NaPi-IIa isoforms, and we observed qualitatively similar behavior (data not shown). Current-voltage (I-V) data pooled from oocytes expressing the flNaPi-IIb were normalized to \(I_P\) at \(V = -100\) mV to take account of different expression levels and aid comparison of the responses for each superfusion condition. As shown in Fig. 2A, 100% replacement of Na\(^+\) with Li\(^+\) gave no significant electrogenic response and this was indistinguishable from the electrogenic response to Pi in 100Ch (data not shown). At hyperpolarizing potentials, replacement of 25, 50, or 75% of external Na\(^+\) with choline caused a concomitant reduction in \(I_P\) compared with 100Na, as we have previously reported (12, 28). The equivalent Li\(^+\) substitution further reduced \(I_P\) compared with the choline case. This was also consistent with the reduced 32P uptake observed for superfusion with 50Na50Li (Fig. 1B).

A comparison of \(I_P\) for the same choline and lithium replacements also revealed that the presence of Li\(^+\) did not simply result in a dose-dependent scaling of the I-V data as might be expected for a simple voltage-independent competitive inhibition of transport function. Instead, we found that increasing the external [Li] also altered the voltage dependence such that the voltage-independent, rate-limiting Pi-induced current normally seen at strong hyperpolarizing potentials now occurred at more depolarizing potentials. For example, with 25Na75Li, \(I_P\) was voltage independent for \(V < -20\) mV (Fig. 2A right), whereas for 50Na50Li, the plateau was reached at ~80 mV (Fig. 2A, middle).

By transposing the data of Fig. 2A at each membrane potential to give the cation activation dose dependence, we compared the effect of equimolar choline (Fig. 2B, left) or lithium (Fig. 2B, right) replacement. In these experiments, the total external monovalent cation strength was maintained at 100 mM. For choline replacement, these data resembled the

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**Fig. 1. Evidence of Li\(^+\) interaction with NaPi-IIa/b from 32P assays. A:** For oocytes expressing flounder type IIA/b Na\(^+\)-coupled inorganic phosphate cotransporters (flNaPi-IIa/b), replacement of Na\(^+\) with Li\(^+\) (open bars) in the uptake medium gave significantly greater uptake (top) than for noninjected (NI) oocytes from the same frog (bottom). Note the 10-fold difference in ordinate scales in A and B. Data pooled from \(n = 9\) oocytes (2 donor frogs). Data were normalized to the mean uptake in 100 mM Na (100Na) for each isoform. B: Comparison of 32P uptake when 50% of Na\(^+\) was equimolarly replaced with choline (50Na50Ch, black bars) or Li\(^+\) (50Na50Li, open bars) for flNaPi-IIb and the rat type IIA isoform (rNaPi-IIa). Data pooled were from \(n = 21\) oocytes from 3 donor frogs and normalized to mean uptake in 50Na50Ch for each isoform.
typical Na⁺ activation for flNaPi-IIb and showed the expected tendency to saturate at high [Na] (5, 12). In contrast, we observed no saturation for equimolar Li⁺ replacement. At low [Na], the relative change in electrogenic response over the voltage range from −160 to 0 mV was smaller when Li⁺ replaced Na⁺ than for choline replacement.

To better understand the underlying mechanism by which Li⁺ affected the transport kinetics, we determined the Pi and Na⁺ activation characteristics with and without a constant [Li] = 50 mM (Fig. 3). We chose this concentration to ensure that the Li⁺ effect was readily detectable and currents were reliably resolved at low [Pi]. Figure 3A compares the pooled I-V data for Pi-activation with a background of 50Na50Ch and 50Na50Li. Two effects of Li⁺ substitution were readily apparent: 1) the voltage dependence was suppressed over the voltage range assayed (−140 to 0 mV) and 2) \( I_p \) showed saturation at a lower [Pi] compared with superfusion in 50Na50Ch. These effects were better visualized by transposing the data (Fig. 3B). We then fit these data with the Michaelis-Menten equation (Eq. 1, with \( n_H = 1 \)) to estimate the maximum Pi-induced current (\( I_p^{\text{max}} \)) and the apparent affinity constant for Pi (\( K_{0.5}^{\text{Pi}} \)).

The predicted \( I_p^{\text{max}} \) plotted as a function of V (Fig. 3C) confirmed the weaker voltage dependence for 50Na50Li for V < 0, and at the hyperpolarizing extreme the maximum predicted electrogenic transport rate was reduced compared with the Li⁺-free case. At strongly hyperpolarizing potentials, the predicted \( K_{0.5}^{\text{Pi}} \) (Fig. 3D) for the three superfusion conditions was similar; however, the data sets deviated from one another as V became more depolarized. For 50Na50Li, \( K_{0.5}^{\text{Pi}} \) decreased with depolarization, which implied that the apparent Pi affinity increased, compared with superfusion with only Na⁺ present, for which we observed the opposite trend.

We next compared the effect of Li⁺ on Na⁺ activation of cotransport by determining \( I_p \) while varying [Na] with a fixed [Li] = 50 mM (Fig. 4). The pooled, normalized, I-V data for this experiment showed the weak voltage dependence for all [Na] and saturation at high [Na] (Fig. 4A). Transposition of these data (Fig. 4B), revealed no inflexion at low [Na] in contrast to the normal behavior for all electrogenic NaPi-IIa/b isoforms when equimolar choline replaced Na⁺ (e.g., Refs. 5, 10, 12, 28). This can be readily seen when the data obtained from a representative oocyte expressing flNaPi-IIb are superimposed (shown here for V = −100 mV; Fig. 4B). The fit using Eq. 1 with free fit parameters gave Hill coefficients (\( n_H \)) that were <1 over the voltage range investigated (Fig. 4C). By constraining \( n_H = 1 \), equally satisfactory fits were obtained and given the variability and range of data points, we were unable to discriminate between the two fit results. This behavior contrasted with that for choline substitution, which showed a clear sigmoidicity and gave \( n_H > 1 \). Moreover, the apparent affinity constant for Na⁺ activation (\( K_{0.5}^{\text{Na}} \)) with 50 mM Li⁺ reported by the fitting algorithm was reduced compared with the Li⁺-free case over the range of test potentials (Fig. 4D). Finally, as expected, the predicted \( I_p^{\text{max}} \) showed less voltage dependence than in the Li⁺-free case (Fig. 4E).

For the complementary experiment (Fig. 5), we maintained [Na] = 50 mM and varied [Li] from 0 to 75 mM. Increasing
external [Li] caused a dose-dependent inhibition of $I_p$ over the entire experimental voltage range (Fig. 5A). The transposed data (Fig. 5B) revealed that at high [Li] the predicted $I_p$ asymptoted to a constant level that depended weakly on $V$. These data were best fit using the Michaelian form of Eq. 1 ($nH/H11005$) with a variable offset to take account of the electrogenic response at [Li]/H11005. The predicted apparent Michaelis constant for the inhibition ($K_{0.5}$) showed a strong voltage dependence and decreased at hyperpolarizing potentials (Fig. 5C).

**Li** Ions and Transport Stoichiometry

The preceding results established that Li$^+$ interacted with NaPi-IIb in the presence of Na$^+$ to alter the maximum transport rate and apparent affinities for Pi and Na$^+$. That significant electrogenic activity remained even for high [Li] indicated that Li$^+$ did not simply compete for occupancy of Na$^+$ interaction sites and thereby block electrogenic, Na$^+$-driven cotransport. One interpretation of these data would be that one or more Li$^+$ ions substituted for Na$^+$ ions and were cotransported, but possibly at a lower turnover rate than with Na$^+$ alone, consistent with our uptake data (Fig. 1A). To test this hypothesis, we performed simultaneous $^{22}$Na uptake and TEVC recording of $I_p$ from individual oocytes as previously described (10, 12, 28) (see MATERIALS AND METHODS). This allowed the determination of the ratio of number of moles of Na$^+$ ($nNa$) to the molar equivalent of charge translocated ($nQt$) for individual oocytes. If indeed Li$^+$ substituted for Na$^+$ as a driving cation, we would predict a reduction in the ratio $nNa:nQt$ (assuming HPO$_4^{2-}$/H11002 remains the preferred substrate). Moreover, this decrease should depend on the relative availability of each cation. On the other hand, if bound Li$^+$ prevented the completion of the cotransport cycle for a given fraction of transporters, $nNa:nQt$ should remain unchanged.

Figure 6 shows the combined results of this assay with a fixed [Na] = 20 mM or 40 mM and with [Li] and [Ch] = 80 mM or 60 mM, respectively. As previously shown (10, 28), when Na$^+$ was the only cation interacting with NaPi-II $nNa:nQt$ was close to 3:1 and this was independent of the external [Na] (Fig. 6A). When Li$^+$ was substituted for choline, there was a significant reduction in $nNa:nQt$ for 40Na60Li from 3.10 ± 0.50 to 2.20 ± 0.13 ($P < 0.01$, by Student’s t-test) and with 20Na80Li the ratio was further reduced from 2.80 ± 0.18 to 1.60 ± 0.23 ($P < 0.004$; Fig. 6B, by Student’s t-test). These findings established that Li$^+$...
reduced $n_{Na^+} - n_{Q^+}$ and indicated that Li$^+$ ions substituted for Na$^+$ ions in the transport cycle.

**Na$^+$ and Li$^+$ Dependence of Presteady-State Kinetics**

The steady-state assays indicated that Li$^+$ ions alter the cotransport voltage dependence and can substitute for Na$^+$ as the cotransported cation species; however, these data offered little insight into which of the three putative cation binding sites were involved or how the kinetics of the partial reactions that represent these interactions were modified. To identify the contributing partial reactions, we investigated the effect of cation substitutions on presteady-state charge movements that

**Fig. 4. Effect of Li$^+$ on Na activation with 1 mM Pi.**

A: I-V showing variation of $I_P$ with $V$ for 6 concentrations of Na$^+$ as indicated and constant 50 mM Li. Data points normalized to $I_P$ at $-100$ mV, in 100Na, 1 mM Pi, and joined for visualization. 

B: transposed data from A fitted with Eq. 1 (continuous lines). 

Data are also shown for a representative oocyte with choline replacement at $V = -100$ mV only, for comparison (○). C: comparison of Hill coefficient ($n_H$) plotted as a function of membrane potential for 0 mM Li$^+$ and 50 mM Li$^+$. Error bars represent SE reported by fit using Eq. 1 to data in B. D: comparison of apparent affinity ($K_{0.5}^{50Li}$) plotted as a function of membrane potential for 0 mM Li$^+$ and 50 mM Li$^+$. Error bars represent SE reported by fit using Eq. 1 to data in B. E: comparison of maximum Pi-induced current ($I_{Pmax}$) for 0 mM Li$^+$ and 50 mM Li$^+$.

**Fig. 5. Li$^+$ inhibition of Pi-activation at 50 mM Na$^+$.**

A: I-V showing dependence of $I_P$ on $V$ for 5 [Li] as indicated. Data points normalized to $I_P$ at $-100$ mV, 100 Na, 1 mM Pi, and joined for visualization. B: transposed data from A fitted with Eq. 1 ($n_H = 1$) with a variable offset to take account of $I_P$ with [Li] = 0. C: comparison of apparent Michaelis constant for inhibition by Li$^+$ ($K_{0.5}^{50Li}$) plotted as a function of membrane potential. Error bars indicate SE reported by fit using Eq. 1.
strong voltage dependence, but no maximum was observed for test potentials up to +80 mV (Fig. 7C, right). For V < -60 mV, although the fitting algorithm reported a slow component, the poor signal-to-noise ratio for hyperpolarizing steps meant that there was greater uncertainty in the fit (Fig. 7C, right).

Figure 8A shows normalized Q-V data obtained from the exponential fit for each of the superfusion conditions. We fit these data with a single Boltzmann function (Eq. 2, MATERIALS AND METHODS) to obtain three phenomenological parameters: Q_{max}, the predicted maximum charge displaced; V_{0.5}, the voltage at which 50% of the charge is displaced; and z the effective valency factor (Fig. 8, B–D).1

To compare the effects of cation substitution in more detail, we normalized the Q-V data to Q_{max} obtained in 100Na and offset each set to superimpose at the depolarizing limit (Fig. 8A). The total detectable mobile charge approximately doubled when Na+ (50 or 100 mM) alone was added to the medium compared with the 100Ch case (Fig. 8B), whereas the addition of Li+ alone (50 or 100 mM) increased Q_{max} to ~70% of that obtained in 100Na. When the superfusate was changed from 50Na50Ch to 100Na, there was a depolarizing shift for V_{0.5} ~25 mV (Fig. 8C), which was consistent with previous reports (12, 29). In contrast, changing from 50Li50Ch to 100Li gave a smaller depolarizing shift in V_{0.5} ~15 mV compared with the Na+ case. Remarkably, replacement of 50% of Na+ with Li+ (50Na50Li) caused a depolarizing shift of ~50 mV relative to the 100Na case (Fig. 8C). This finding provided further support for the notion that Li+ ions interacted with the flNaPi-Iib protein and altered the voltage-dependent kinetics. Moreover, although we were unable to apply voltage steps beyond +80 mV because of contamination of the records from the activation of endogenous Cl− channels, the Q_{max} prediction from the Boltzmann fits strongly suggested that this parameter remained reasonably constant for the three conditions: 100Na, 50Na50Ch, and 50Na50Li (Fig. 8B). Finally, the apparent valency (z) increased over that of the empty carrier value (0.40 ± 0.04) when Na+ or Li+ or both Na+ and Li+ were present in the superfusate (Fig. 8D). With only Li+ present, z increased to 0.52 ± 0.04, whereas when Na+ was present it increased from 0.56 ± 0.02 (50Na50Ch) to 0.65 ± 0.02 (100Na).

We also investigated if other monovalent cations could induce similar effects: we made 100% substitutions with Rb+, Cs+, and K+, however, no additional charge movement was observed (Fig. 8E). This suggested that the size of the cation was of critical importance for interactions with flNaPi-Iib as Na+ and Li+ have comparable but smaller atomic radii compared with the other cations investigated (e.g., Ref. 16).

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1 Given the significantly different time constant associated with the 50Na50Li superfusion, we took pains to ensure that this was not an artifact that might arise, for example, from slow activating endogenous currents. To this end, we confirmed that the charge movement in the presence of Li+ fulfilled three properties expected for a fixed number of mobile charges that can move within the transmembrane electric field and are directly associated with functionally expressed flNaPi-Iib. These properties were as follows: 1) the total charge displaced was proportional to the I_{0V}, 2) the charge movement was reversible over the test voltage range, i.e., the magnitude of charge induced by the ON step (from V_{0} to the test potential) and OFF step (from the test potential to V_{0}) were in good agreement; and 3) the charge movement was the result of a memoryless process; i.e., by changing V_{0} to +40 mV, the Q-V was displaced along the Q axis and, moreover, yielded comparable estimates of the Boltzmann parameters (Q_{max}, V_{0.5}, z; data not shown).

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Fig. 6. Lithium alters flNaPi-Iib stoichiometry. A: relationship between net charge transfer (Q), and radiolabeled Na+ uptake for oocytes expressing the flNaPi-Iib isofrom and voltage clamped at −50 mV in the presence of 40 and 20 mM Na+ together with 60 mM and 80 mM choline, respectively. Both Q, and Na+ uptake were expressed as their molar equivalents (nNa/nCh). Each point was obtained from a single oocyte. Representative data sets were fitted with a linear regression line with variable slope and y-intercept to account for endogenous uptake in control oocytes into account. Slopes were not significantly different and support nNa/nCh stoichiometry of 3:1, as previously reported (10). B: relationship between Q, and radiolabeled Na+ uptake as in A in the presence of 40 and 20 mM Na+ together with 60 and 80 mM Li, respectively. Each point was obtained from a single oocyte. Linear regression fitting as in A. Slopes were statistically different from each other (P < 0.05, by Student’s t-test) and indicated a reduction of stoichiometry due to the presence of Li+ in the medium. Gray dotted line represents the ratio equal to 3.

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Fig. 7 shows representative original records membrane current relaxations under different superfusion conditions for voltage steps from a −60-mV holding potential (V_{h}) to test voltages in the range of −180 to +80 mV. For a representative noninjected oocyte, this protocol induced transient current relaxations that typically lasted 1–2 ms with low-pass filtering at 500 Hz (Fig. 7A), which, when integrated, showed a linear dependence on membrane potential (not shown). For a representative flNaPi-Iib-expressing oocyte from the same donor and Na+ and Li+ uptake were expressed as their molar equivalents (nNa/nCh). Each point was obtained from a single oocyte. Representative data sets were fitted with a linear regression line with variable slope and y-intercept to account for endogenous uptake in control oocytes into account. Slopes were not significantly different and support nNa/nCh stoichiometry of 3:1, as previously reported (10). B: relationship between Q, and radiolabeled Na+ uptake as in A in the presence of 40 and 20 mM Na+ together with 60 and 80 mM Li, respectively. Each point was obtained from a single oocyte. Linear regression fitting as in A. Slopes were statistically different from each other (P < 0.05, by Student’s t-test) and indicated a reduction of stoichiometry due to the presence of Li+ in the medium. Gray dotted line represents the ratio equal to 3.

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we have previously shown reflect voltage-dependent transitions in the transport cycle (5, 7, 9, 11, 12).

Figure 7 shows representative original records membrane current relaxations under different superfusion conditions for voltage steps from a −60-mV holding potential (V_{h}) to test voltages in the range of −180 to +80 mV. For a representative noninjected oocyte, this protocol induced transient current relaxations that typically lasted 1–2 ms with low-pass filtering at 500 Hz (Fig. 7A), which, when integrated, showed a linear dependence on membrane potential (not shown). For a representative flNaPi-Iib-expressing oocyte from the same donor frog and superfusion in 100Na, 100Ch, and 100Li, respectively, voltage steps gave rise to relaxations that were superimposed on the endogenous transients (Fig. 7B). The presence of relaxations for superfusion in 100Li suggested that, like Na+, Li+ ions were able to interact with the expressed protein in a voltage-dependent manner. We also observed relaxations if the oocyte was superfused in 50:50 mixtures of the superfusates. Remarkably, for the case of superfusion in 50 mM NaCl + 50 mM LiCl (50Na50Li), the relaxations for voltage steps to potentials >0 were significantly slower than those in the absence of Li+.

To quantify these observations, we determined the charge (Q) associated with flNaPi-Iib by fitting the relaxations to exponentially decaying functions (see MATERIALS AND METHODS). For superfusion in Na+ and choline, the relaxations were well described by fitting a single exponential to the relaxation after the linear capacitive component was complete (see MATERIALS AND METHODS). However, when both Li+ and Na+ were present, we found that a double exponential fit described more accurately the current decay (see MATERIALS AND METHODS). The faster time constants (Fig. 7C, left) were typically <10 ms over the test voltage range and showed a “bell-shaped” dependence on V as previously reported for the flNaPi-Iib (5–7). The slow time constant observed for superfusion with 50Na50Li showed
Fig. 7. Li⁺ substitution and presteady-state charge movement. A: voltage-step protocol used for inducing presteady-state charge relaxations (left) and response of a representative noninjected oocyte (right). For this oocyte, large positive voltages also evoked a response from endogenous Cl⁻ channels. Maximum and minimum of each capacitive charging transient have been clipped graphically. B: current traces recorded from a representative oocyte expressing flNaPi-IIb (from same donor frog as in A) in response to the protocol in A under 6 superfusion conditions: top: 100 mM NaCl (100Na), 100 mM LiCl (100Li), and 100 mM choline Cl (100Ch); and bottom: 50 mM NaCl/50 mM cholineCl (50Na50Ch), 50 mM LiCl + 50 mM cholineCl (50Li50Ch), and 50 mM NaCl + 50 mM LiCl (50Na50Li). C: voltage dependence of time constants obtained from fitting the ON relaxations (steps from Vₖ = −60 mV to test potential) in B to single (100Na, 50Na50Ch) and double (50Na50Li) exponential functions. Note the different scales for the 50Na50Li data. Each data point is means ± SE for 6 oocytes. Data points are joined to aid visualization.

Qualitatively similar behavior was observed for the human and rat NaPi-IIa isoforms, which confirmed that the effect of Li⁺ on the voltage dependence of presteady-state kinetics was not unique to the WT flNaPi-IIb (data not shown). Moreover, the generality of the Li⁺ effect was demonstrated using a mutant flNaPi-IIb construct (A175C) that shows a significantly altered voltage dependence compared with the WT (14). For superfusion in 100Na, its presteady-state voltage dependence is shifted towards hyperpolarizing potentials compared with the WT, and, like the WT, superfusion with 50Na50Li caused a 50 mV depolarizing shift in V₀.5 relative to the estimate in 100Na (Fig. 8, F and G). These findings suggested that the effect of Li⁺ ions was the same in both cases and supported the notion that like Na⁺ ions, Li⁺ ions bind to one or more sites located within the transmembrane electric field (9).

To gain further insight into the Li⁺ interactions, we performed a detailed analysis of the presteady-state charge movements with varying [Li] in the presence and absence of fixed [Na] and the converse. Figure 9 shows the variation of Boltzmann parameters for varying cation concentrations with equimolar choline substitution. For Qₘₐₓ (Fig. 9A), all fit estimates were normalized to the predicted Qₘₐₓ at 100Na for each oocyte and then pooled. This fit parameter remained reasonably constant for [Na] or [Li] > 10 mM and the maximum displacable charge in the presence of Li⁺ remained at ~70% of that obtained in Na⁺. Both z (Fig. 9B) and V₀.5 (Fig. 9C) increased
At high cation concentrations, replotted on a log10 concentration. This became more evident when these data were for either cation, we observed a significant difference in $V_{0.5}$ plotted as a function of the respective cation concentration. This became more evident when these data were replotted on a log10 scale, where $X = Na^+$ or $Li^+$. At high cation concentrations, $V_{0.5}$ then varied linearly and the limiting slopes were 118±8 and 64±3 mV/decade for $Na^+$ and $Li^+$, respectively. For $[Li] < 25$ mM, $V_{0.5}$ deviated from the linear behavior and showed less dependence on $[Li]$. In the final series of experiments, we repeated these measurements and varied one cation in the presence of a constant concentration (10 and 50 mM) of the other (Fig. 10). For variable $[Na]$, $Q_{max}$ normalized to the value estimated for each oocyte at 100Na, was close to unity for $[Li] = 10$ mM and 50 mM (Fig. 10A, left), whereas for variable $[Li]$ it depended on $[Na]$. We attributed the large error in normalized $Q_{max}$ with $[Na] = 50$ mM and variable $[Li]$ to the lack of saturation of the $Q-V$ at depolarizing potentials for this condition (see Fig. 8A). This also had consequences for the uncertainty in predictions for $z$ and $V_{0.5}$. By constraining the fits with a fixed $Q_{max}$ obtained from the 100Na fit for each oocyte, the Boltzmann fits to the $Q-V$ data for the variable $[Na]$ case were not compromised and the respective uncertainties in $z$ and $V_{0.5}$ were reduced. The apparent valence ($z$) for both variable $[Na]$ and $[Li]$ (Fig. 10B) showed a small concentration-dependent increase. Moreover, $V_{0.5}$ for all conditions showed a characteristic linear dependence on log10[$Na$] or log10[$Li$]. For $Na^+$ as the variable cation, the limiting slopes in the presence of external $Li^+$ were significantly smaller (56 ± 4 mV/decade for 10 mM $Li^+$ and 58 ± 6 mV/decade for 50 mM $Li^+$) compared with the slope in the absence of external $Li^+$ (118 ± 8 mV/decade). Moreover, the intercept on the $[Na]$ axis shifted to the left as $[Li]$ increased. In contrast, the addition of a fixed $[Na]$ to the external medium with variable $[Li]$ (Fig. 10C, right), the slopes were as follows: 64 ± 3 mV/decade (0 mM $Na^+$), 69 ± 4 mV/decade (10 mM $Na^+$), and 68 ± 3 mV/decade (50 mM $Na^+$). For this experimental protocol, the intercept on the $[Li]$ axis also shifted to the left as $[Na]$ increased. This apparent reciprocity of the influence of one cation species on the voltage dependence of the other cation.
suggested a reciprocal interaction between Na\(^+\) and Li\(^+\) ions competing for occupancy of the same site(s) in the protein.

**DISCUSSION**

**Stoichiometry Assays Establish That Li\(^+\) Can Substitute for Na\(^+\) in the Cotransport Cycle**

The correlation between charge translocated (\(Q_t\)) and Na\(^+\) uptake under voltage clamp offered compelling evidence that Li\(^+\) ions can substitute for Na\(^+\) as a driving cation. Although there was experimental scatter in the data, equimolar Li\(^+\) substitution for choline resulted in a statistically significant reduction in the molar stoichiometry ratio (\(n^{Na}/n^{Li}\)), which would be expected if Li\(^+\) replaced one or more Na\(^+\) ions in the transport cycle. It is tempting to conclude from these data that the Li\(^+\) ion had substituted for one of the 3 Na\(^+\) ions cotransported. However, uncertainties in the data do not allow us to rule out that >1 Na\(^+\) ion was replaced by Li\(^+\). Indeed, the noninteger stoichiometry that we observed at the macroscopic level would be expected from a mixed population of transporters operating with \(n^{Na}/n^{Li}\) = 3, 2, or 1, depending on the occupancy of cation binding sites on a cycle-to-cycle basis. Despite these uncertainties, the results of the stoichiometry assay allowed us to rule out two hypotheses for the mechanism of Li\(^+\) interaction: First, if Li\(^+\) suppressed electrogenic cotransport but the protein completed the transport cycle in an electroneutral mode, we would expect \(n^{Na}/n^{Li}\) > 3:1 obtained for superfusion with only Na\(^+\) present. Second, if Li\(^+\) competitively inhibited cotransport without contributing net charge translocation, no change would be expected.

**Does Li\(^+\) Alone Act as a Driving Cation?**

Uptake assays in which we replaced all external Na\(^+\) with Li\(^+\) suggested that Li\(^+\) may also drive Pi, cotransport, albeit at a much lower turnover rate than for Na\(^+\) (Fig. 1A). The low uptake activity precluded a more detailed kinetic analysis to determine if this resulted from a reduced maximum transport rate, a reduced substrate apparent affinity (Li\(^+\) or Pi), or a combination of both. It might also be argued that the \(^{32}\)P uptake measured in 100Li was the result of transport via endogenously expressed proteins from the SLC20 family (e.g., PiT-1,2), given that in oocytes overexpressing the Xenopus PiT-1 a resolvable electrogenic response was found in 100 mM Li\(^+\). In the present study, there was indeed a significantly higher uptake in 100Li compared with 100Ch for noninjected oocytes, which may suggest the involvement of endogenous PiT; however, the overall levels were 10-fold lower than for oocytes expressing NaPi-IIb.

Based on the uptake data, we predict that voltage clamped oocytes expressing fNaPi-IIb with typical P\(_I\)-induced currents (\(I_P\) \(\sim\) -200 nA at \(-100 \text{ mV} \) (1 mM P\(_I\))) would give correspondingly small \(I_P\) \(\sim\) -6 nA in 100Li. However, by TEVC, we were unable to detect a significant inward current in the presence of 100 mM Li\(^+\). Although currents in the nanoampere range are resolvable with the TEVC, we routinely observed that noninjected oocytes also display an electrogenic response to P\(_I\) within this range, which appears to be batch dependent. This is thought to be related to endogenous chloride channels (I. C. Forster, unpublished observations) and could easily mask any NaPi-IIb-dependent P\(_I\) response in 100 mM Li\(^+\). On the other hand, we obtained evidence that Li\(^+\) ions alone can specifically interact with NaPi-IIb from the presteady-state assays in this study (see below) as well as from previous voltage-clamp fluorometry studies on both NaPi-IIb (29, 30) and the electroneutral NaPi-IIc (15). More convincing evidence for the role of Li\(^+\) as a driving cation was obtained from assays performed in the presence of Na\(^+\).

**Interactions of Li\(^+\) in the Presence of Na\(^+\): Steady-State Kinetics**

The altered steady-state kinetics when external Na\(^+\) was replaced with Li\(^+\) indicated that Li\(^+\) ions influence one or more partial reactions in the transport cycle that determine the overall voltage dependence of transport, as well as the turnover rate (cotransport rate) of the protein. An additional indication that membrane potential-dependent partial reactions are implicated comes from work on the electroneutral isoform NaPi-IIc (15). This protein shows no steady-state or presteady-state charge movement, and \(^{32}\)P uptake is voltage-independent, implying that no voltage-dependent partial reactions contribute to the transport cycle. In that study, we reported that there was no significant change in uptake when 50% of Na\(^+\) was replaced with Li\(^+\). This suggested that the Li\(^+\) effects reported in the present study were unique to the electronegic NaPi-IIa/b...
and implied an interaction of Li$^+$ ions with voltage-dependent partial reactions that precede Pi binding.

It was previously reported for the Na$^+/H^+$-coupled succinate transport (NADC-1; Ref. 24) that Li$^+$ ions, in the presence Na$^+$ ions, caused a mixed type inhibition to account for the reduced apparent succinate affinity and reduced maximum transport rate for that transporter. These effects of Li$^+$ on NaDC-1 were observed at relatively low [Li] (5 mM). For flNaPi-IIb, our findings are also not consistent with a classical competitive inhibition of transport by Li$^+$ for which we would expect a decrease in the apparent substrate affinities and an unchanged maximum transport rate (see Fig. 3, C and D). For the Na$^+$-activation experiments with fixed [P], the apparent affinity for Na$^+$ increased in the presence of 50 mM Li$^+$ compared with choline substitution (Fig. 4D). Moreover, these experiments revealed that the normally sigmoidal behavior observed for Na$^+$ activation with choline replacement was absent: fitting with the Hill equation predicted a Hill coefficient $n_H < 1$, which indicated negative cooperativity, although in practice a Michaelian fit ($n_H = 1$) was also acceptable. This would be consistent with Li$^+$ ions replacing one or more Na$^+$ ions in the transport cycle. Alternatively, the reduced cooperativity of the Na$^+$ interactions could be explained by Li$^+$ ions modulating the transport cycle without functioning as a driving cation. With a constant [Na] = 50 mM and variable [Li], activation of $I_P$ (Fig. 5B) showed a Li dependence that could also be described by a Michaelian function, which suggested that one Li$^+$ ion interacted with the protein. These data provided further confirmation that Li$^+$ does not act as a simple competitive inhibitor of Pi transport, because as [Li] increased, the transport rate reached a plateau that was weakly voltage dependent.

This behavior suggested that the protein operates using Na$^+$ ions alone with a high transport turnover rate or with both Li$^+$ and Na$^+$ ions at a lower turnover rate. The mixed cation cycle would progressively dominate as [Li] increased until a limiting transport rate is reached. Moreover, the Michaelian behavior for Li$^+$ interaction was consistent with voltage clamp fluorom-
etry (VCF) data obtained from the same isoform (30), which indicated that one Li⁺ ion interacted with the protein in the absence of Pi. For NaDC-1, similar behavior was reported whereby the inhibition by Li⁺ was best described by a Michaelian function and the maximal inhibition ranged from 54% at -150 mV to 76% at -10 mV (24).

Interactions of Li⁺ in the Presence of Na⁺: Presteady-State Kinetics

Steady-state assays alone do not allow an unambiguous identification of which partial reactions are Li⁺ dependent because the electrogenic response is a function of all partial...
reactions in the cotransport cycle. Furthermore, we cannot exclude the possible involvement of electron-neutral partial reactions. We gained further insight by restricting the allowed partial reactions in the transport cycle to those that precede Pi interaction and investigated their presteady-state kinetics.

In the absence of external alkali metal ions (100Ch), presteady-state charge movements were detected that we attribute to voltage-dependent molecular rearrangements of the empty carrier (e.g., Ref. 9). With the addition of either Na\(^+\) (100Na) or Li\(^+\) (100Li), the total detectable charge movement (\(Q_{\text{max}}\)), predicted from fitting a single Boltzmann function to the \(Q-V\) data, increased, which indicated that these cations enter the transmembrane electric field (Fig. 8B) and contribute to net transmembrane electric field (Fig. 8B) and contribute to net charge movement.\(^2\) However, we found that \(Q_{\text{max}}\) for 100Li superfusion was \(\approx 70\%\) of that obtained for 100Na. One mechanistic interpretation of this finding is that for superfusion in 100Li, Li\(^+\) ions move across a smaller fraction of the transmembrane electric field to reach their binding site compared with Na\(^+\) ions. This would also be consistent with the Boltzmann fit estimate of the apparent valence (\(z\)) for 100Li being less than that found for 100Na or 50Na50Ch (Fig. 8D). When both Na\(^+\) and Li\(^+\) were present in the external medium (50Na50Li), \(Q_{\text{max}}\) was similar to that obtained with superfusion in 100Na. This suggested that the charge contribution due to the Li\(^+\) alone did not arise from Li\(^+\) interacting with the protein at an independent site, for if that were the case, we would expect a larger \(Q_{\text{max}}\) than for superfusion in 50Na50Ch.

Another feature of the presteady-state behavior that was steady-state mobile charge distribution (\(Q-V\)) was significantly affected by the cation species present in the external medium (Fig. 8A). We quantified this in terms of the midpoint voltage \(V_{0.5}\) at which 50\% of the charge was displaced (Fig. 8C). These findings provided further evidence of Na\(^+\) and Li\(^+\) ions interacting at one or more common sites within the transmembrane electric field.

2 The 100Li result contrasts with our previous study (30) on the fNaPi-IIb mutant S448C in which no change was reported and might be attributable to the effect of the cysteine mutation on cation interactions or to more precise estimation of charge movement possible from WT expressing oocytes in the present study that were selected with \(|dE| = 400\, \text{nA} (1\, \text{mM} \text{P})\).

Semilog plots of \(V_{0.5}\), as a function of cation concentration revealed significantly different limiting slopes at high cation concentrations: \(\approx 60\, \text{mV/log}_{10}[\text{Li}]\) and \(\approx 120\, \text{mV/log}_{10}[\text{Na}]\). By assuming that the total net charge movement across the whole membrane field = 1, these slopes are consistent with 2 Na\(^+\) ions or 1 Li\(^+\) ion, respectively, interacting with the empty carrier, according to a three-state model that comprises an empty carrier reorientation followed by simultaneous cation binding (see APPENDIX). However, this three-state model cannot readily account for our finding that \(Q_{\text{max}}\) for 100Li was \(\approx 70\%\) of \(Q_{\text{max}}\) for 100Na.

By extending the model to a 4-state scheme (see APPENDIX), comprising the empty carrier partial reaction followed by two sequential cation interactions, only the first of which is assumed to involve Li\(^+\), we can indeed account for the presteady-state experimental findings. Moreover, this lends support to our previous predictions from voltage clamp fluorometry that were consistent with 2 Na\(^+\) ions or 1 Li\(^+\) ion interacting with the transporter.

For the four-state model, the limiting slope for Na\(^+\) as the variable cation is \(\approx 116/(z_{\text{01}} + z_{\text{12}} + z_{\text{23}})\, \text{mV/log}_{10}[\text{Na}]\) at \(20^\circ\text{C}\), where \(z_{\text{01}}\) is the apparent valence of the empty carrier and \(z_{\text{12}}, z_{\text{23}}\) are the effective valences of the respective Na\(^+\) interactions. Assuming one net charge crosses the membrane field, i.e., \(z_{\text{01}} + z_{\text{12}} + z_{\text{23}} = 1\), the limiting slope = 116 mV/log\(_{10}\) [Na], which is close to \(118\, \text{mV/log}_{10}\) [Na] found experimentally. With only Li\(^+\) present, one cation interaction occurs and the model reduces to the three-state case in which one Li\(^+\) ion would bind, for which we predict a slope of 58 mV/log\(_{10}\) [Li], close to \(64\, \text{mV/log}_{10}\) [Li] found experimentally. This sequential model also predicts that the ratio of maximum mobile charge displaced for Li\(^+\) vs. Na\(^+\) (\(Q_{\text{max}}^{\text{Li}+/\text{Na}^+}\)) is \(z_{\text{01}}/(1 + z_{\text{12}} + z_{\text{23}})\), so if we take the apparent valence for the empty carrier \(z_{\text{01}} = 0.4\) (Fig. 8D), then for the experimentally obtained ratio \(Q_{\text{max}}^{\text{Li}+/\text{Na}^+}\) = 0.7, and \(z_{\text{12}} = z_{\text{23}} = 0.3\).

We obtained further insight into the cation interactions by determining the Boltzmann parameters from the \(Q-V\) data when we varied one cation and fixed the other cation (Fig. 10). This behavior of \(V_{0.5}\) vs. \(\log_{10}[X] (X = \text{Na or Li})\) can be
understood in terms of a six-state model for cation interaction in which the empty carrier is either loaded with two Na⁺ ions or one Li⁺ ion followed by a Na⁺ ion (see APPENDIX). For a population of carriers, the occupancy of the putative cation binding sites at any given membrane potential depends on the activity of the cations and the ratios of the forward to backward rates for each partial reaction. For both sets of data, an increasing concentration of the fixed cation shifted the intercept on the log₁₀[X] axis to the left. This intercept can be thought of as an apparent affinity for the variable cation when the membrane potential is zero (e.g., Ref. 22). The left shift therefore indicated that the apparent affinity for the variable cation increased as the fixed cation concentration increased.

The observed reciprocal behavior would not be expected if both cations simply competed for the same binding site, but rather suggests a cooperative interaction, whereby the binding of one cation increases the apparent affinity for the other. With appropriate choice of parameters, the six-state model predicts the experimentally observed behavior of Vₐₜ₀ as shown in Fig. 10C. Some discrepancy between predicted and experimental data, especially at low concentrations, most likely arises from the fact that the experimental values were derived from a single Boltzmann fit to the data, whereas in the Q-V function derived for the model is a more complex function (see APPENDIX).

Extended Kinetic Scheme for Electrogenic NaPi-II Transporters

To account for the present findings, we propose an extension to our current kinetic scheme (e.g. Ref. 14) by incorporating a second cotransport cycle: this operates when Li⁺ occupies the first cation binding site (Fig. 11A). The partial reaction rates for the second Na⁺ interaction and translocation rates for the fully loaded carrier distinguish the kinetics of each cycle. In agreement with the presteady-state analysis, we assume that Li⁺ can only bind at the first cation binding site that becomes available when the empty carrier occupies state 1. If Li⁺ is bound, a second Na⁺ ion can bind cooperatively and cotransport proceeds with 2 Na⁺ ions and 1 Li⁺ ion cotransported with Pᵢ. This accounts for the reduced NaᵢQᵢ stoichiometry found experimentally. The probability of this cycle occurring depends on the relative activities of Li⁺ and Na⁺ in the external medium. If Li⁺ is the only monovalent cation in the external medium, we assume cotransport would be unfavorable because subsequent Li⁺ ions bind with very low affinity. This case, not shown in the scheme, could account for the very low uptake reported in 100Li. In the absence of external Na⁺ and Pᵢ, we predict that Li⁺ can preferentially only occupy the first cation binding site and the probability of a second Li⁺ binding is very low. This accounts for the lower Qₘₐₓ observed and the reduced z compared with having Na⁺ also present. Thus the kinetics of the presteady-state relaxations obtained in 100Li reflect the voltage-dependent interactions of a single cation with the carrier.

To test the behavior of the model under presteady-state and steady-state conditions, we used the ratios for the forward to backward rates of the voltage-dependent partial reactions at V=0 (α₀, β₀, γ₀, δ₀, ε₀) that were obtained by matching the predictions from the six-state scheme (see APPENDIX) to the experimental data. We then scaled the absolute rates to simulate presteady-state relaxations that gave a reasonable match to the experimental data. Thus the characteristic slow ON relaxations for a depolarizing voltage step observed with superfusion in 50Na50Li arise from the slow backward rate k₂⁻→₁ = 40 s⁻¹ associated with debinding of the second Na⁺ ion that precedes Li⁺ debinding. As shown in Fig. 12B, the simulated Q-V data for different compositions of the external media (in the absence of external Pᵢ) satisfactorily recapitulate the shifts in charge distributions observed in the measured data (Fig. 8A).

To account for the steady-state behavior, we sought the minimum number of changes in rates for the remaining electroneutral partial reactions in the Li-Na cycle to account for the experimental data. In this respect, it was necessary to reduced rates associated with Pᵢ debinding (3'→2') in the Li-Na cycle and the final translocation rate (4'→5') compared with the Na-Na cycle. On the other hand, good agreement between measurement and model behavior was obtained by keeping the rates of the final Na interaction the same for both cycles. The model and complete parameter set (Fig. 11) also successfully accounted for the steady-state behavior with varying substrates and reproduced the essential features observed experimentally for Pᵢ, Na, and Li activation. For example, the simulations (Fig. 11C) recapitulate the differences in the steady-state Na-activation kinetics, depending on whether choline or Li⁺ was used to replace Na⁺ (Fig. 2B).

Apart from the obvious shift in the voltage dependence caused by Li, its main effect on the steady-state kinetics was to reduce the cooperativity of Na-activation (Fig. 4B). This can be understood in terms of the altered zero voltage rate ratio factors for the second Na⁺ binding partial reaction (i.e., γ₀ = 40 M⁻¹ in the absence of Li compared with ε₀=500 M⁻¹ in the presence of Li). For the Na-Na-Pᵢ-Na cycle, positive cooperativity results from having β₀ < γ₀ < ε₀, where γ₀ = k₄/₅/k₅₄ = k₄₅/k₅₄ is the ratio factor for the final Na⁺ interaction (=400 M⁻¹ in the model simulation). In contrast, for the Li-Na-Pᵢ-Na cycle, γ₀ > ε₀, which explains the apparent negative cooperativity for Na⁺ interaction. The weaker dissociation of the second Na⁺ ion when Li⁺ is bound also increases the apparent affinity for Pᵢ.

![Fig. 12. Cation binding schemes. A: 3-state scheme representing empty carrier (0→1) and simultaneous cation binding (1→2). B: 4-state scheme in which cations bind sequentially (1→2, 2→3) to the empty carrier. C: 6-state scheme in which one Li⁺ ion competes with Na⁺ for occupancy of the first cation binding site, followed by binding of a second Na⁺ ion.](http://ajpcell.physiology.org/doi/10.1152/ajpcell.00364.2011)
The overall cotransport activity from a population of transporters results from contributions from both cycles. This depends on the probability of a Na$^+$ or Li$^+$ ion binding first. This can be visualized by examining the occupancies of states 5 and 5$'$ (Fig. 11C), which reflect the relative proportion of transporters that are available to translocate P$_i$ together with either 3 Na$^+$ ions or a Li$^+$ ion substituted for the first Na$^+$ ion. For the case where Na$^+$ is the only cation present, the occupancy of state 5 is a strong function of membrane potential and a fixed total cation concentration (Fig. 11C, left). If, however, we have an equimolar substitution of Li$^+$ ions for Na$^+$ ions, and by assuming saturating P$_i$ (1 mM), the model predicts that the transporters will be mainly distributed between states 5 and 5$'$ (Fig. 11C, right). This distribution between states 5 and 5$'$ is a strong function of [Na], [Li], and V. The weaker voltage dependence for state 5$'$ is readily seen and, moreover, the presence of Li$^+$ ions markedly alters how voltage affects the occupancy of state 5.

Conclusions

The present study has demonstrated that Li is a useful probe to investigate cation interactions with an electrogenic cotransporter, NaPi-IIb. In particular, our findings provide strong functional evidence for sequential, cooperative interaction of 2 Na$^+$ ions with the empty carrier before P$_i$ binds. By interpreting the behavior of macroscopic charge movements that result from cation movement within the transmembrane electric field, together with charges intrinsic to the protein, in terms of a simple transition state model, we provide a useful analytical platform for studying multiple cation interactions with other electrogenic transporters. Although the underlying molecular mechanisms cannot be currently elucidated at the protein structural level without a three-dimensional model of NaPi-II proteins (or their bacterial homologs), we predict that the smaller size of Li$^+$ compared with Na$^+$ will be critical to explain the observed behavior.

APPENDIX

Models for Cation Interactions Based on Eyring Rate Theory

Three-state model incorporating the empty carrier and simultaneous cation binding. For a three-state model (Fig. 12A), in which transition 0$\rightarrow$1 represents the empty carrier and transition 1$\rightarrow$2 represents the simultaneous binding of $n$ monovalent cations (e.g., Na$^+$) and both partial reactions contribute to charge movement, the rate constants using Eyring rate theory (e.g., Ref. 16) and assuming symmetrical barriers are given by:

$$
\begin{align*}
    k_{01} &= k_{01}^0 \exp(-z_{01} V_e/2kT) \\
    k_{10} &= k_{10}^0 \exp(z_{01} V_e/2kT) \\
    k_{12} &= \left[N_a^+\right]k_{12}^0 \exp(-z_{12} V_e/2kT) \\
    k_{21} &= k_{21}^0 \exp(z_{12} V_e/2kT)
\end{align*}
$$

where $z_{01}$ and $z_{12}$ are the valences of the equivalent charge that would be displaced across the whole membrane field associated with the respective partial reactions, $k_{01}^0$ is the rate for transition $i \rightarrow j$ when $V = 0$, where i and j are the respective states, $[Na^+]$ is the Na$^+$ concentration, and $e$, $k$, and $T$ have their usual meanings. The transient current $i_{pas}(t)$ that accompanies a change of state for $N_i$ transporter molecules is given by:

$$
i_{pas}(t) = e \left[ (z_{01} + z_{12}) \frac{dN_0}{dt} + z_{12} \frac{dN_1}{dt} \right]
$$

where $N_i$, (i = 0 . . . 2) refer to the population of the states and $\Sigma N_i = N$, and

$$
\begin{align*}
    \frac{dN_0}{dt} &= N_i k_{10} - N_0 k_{01} \\
    \frac{dN_1}{dt} &= N_0 k_{01} + N_2 k_{21} - N_1 (k_{10} + k_{12})
\end{align*}
$$

The charge displaced by a voltage step from $+\infty$ (all transporters in state 0) to a test potential, V, is found by integration of Eq. A2 over time:

$$
Q_V^\infty = -N_i e \left\{ \frac{\alpha \beta (z_{01} + z_{12} + z_0 z_1 \alpha)}{1 + \alpha + \alpha \beta} \right\}
$$

In the limit for [Na] large, Eq. A5 simplifies to:

$$
\alpha^0 \left[N_a^+\right] \beta^0 (e^{-eV_0过过z_{12}V_e}) = 1
$$

where $\alpha^0 = k_{01}/k_{10}$ and $\beta^0 = k_{12}/k_{21}$. It follows that a plot of $V_0$ vs. $\ln [Na]$ yields a limiting slope = $kT e(z_{01} + z_{12})$ [mV/e-fold change in [Na]] or $\approx 58 (z_{01} + z_{12})$ mV/10-fold change in [Na] at 20°C. It is important to note that, if $z_0 + z_1 = n$, the limiting slope will be independent of n (58 mV/10-fold [Na]).

Four-state model incorporating the empty carrier and sequential cation binding. The four-state model (Fig. 12B) incorporates an additional cation binding step (2$\rightarrow$3). We assume that the partial reactions 1$\rightarrow$2 and 2$\rightarrow$3 represent the interaction of one cation each. Following the same procedure as above, for a voltage step from $+\infty$ (all transporters in state 0) to a test potential V, the charge displaced is given by:

$$
Q_V^\infty = -N_i e \left\{ \frac{(z_{01} + z_{12} + z_{23}) + (z_{12} + z_{23}) \alpha + z_2 z_3 \beta}{1 + \alpha + \alpha \beta + \alpha \beta \gamma} \right\}
$$

In the limit, for [Na] large, Eq. A8 simplifies to:

$$
\alpha^0 \beta^0 (e^{-eV_0过过z_{12}V_e}) = 1
$$

where $\alpha^0 = k_{01}/k_{10}$, $\beta^0 = k_{12}/k_{21}$, and $\gamma^0 = k_{23}/k_{32}$. A plot of $V_0$ vs. In [Na] yields a limiting slope = $2kT e(z_{01} + z_{12} + z_{23})$ [mV/e-fold change in [Na]] or $\approx 116 (z_{01} + z_{12} + z_{23})$ mV/10-fold change in [Na] at 20°C.

For one net charge translocated across the transmembrane field, the predicted slope is then 116-mV/10-fold change in [Na].

Six-state model incorporating the empty carrier and different cations. The six-state model (Fig. 12C) takes account of sequential binding of two Na$^+$ ions or one Li$^+$ ion followed by a Na$^+$ ion, which leads to a mixed population of transporters in two unique states, 3 or 3$, depending on the intrinsic rates and cation concentration as $V \rightarrow -\infty$. We assume that each partial reaction that involves cation binding represents the interaction of one cation only. For a voltage step from $\approx$ (all transporters in state 0) to a test potential V, the charge displaced is given by:

$$
i_{pas}(t) = e \left[ (z_{01} + z_{12}) \frac{dN_0}{dt} + z_{12} \frac{dN_1}{dt} \right]
$$

where $N_i$, (i = 0 . . . 2) refer to the population of the states and $\Sigma N_i = N$, and

$$
\begin{align*}
    \frac{dN_0}{dt} &= N_i k_{10} - N_0 k_{01} \\
    \frac{dN_1}{dt} &= N_0 k_{01} + N_2 k_{21} - N_1 (k_{10} + k_{12})
\end{align*}
$$

The charge displaced by a voltage step from $+\infty$ (all transporters in state 0) to a test potential, V, is found by integration of Eq. A2 over time:

$$
Q_V^\infty = -N_i e \left\{ \frac{\alpha \beta (z_{01} + z_{12} + z_0 z_1 \alpha)}{1 + \alpha + \alpha \beta} \right\}
$$

where $\alpha = k_{01}/k_{10}$ and $\beta = k_{12}/k_{21}$ and $k_0$ are given by Eq. A1. The voltage at which 50% of the total charge has moved (V$_{0.5}$) is found by solving Eq. A4 for V with $Q = 0.5 Ne \left( z_{01} + z_{12} \right)$. Equation A4 reduces to:

$$
\left[ (z_{01} + z_{12}) (\alpha + \alpha \beta - 1) - 2a z_{12} \right] = 0
$$

In the limit for [Na] large, Eq. A5 simplifies to:

$$
\alpha^0 \left[N_a^+\right] \beta^0 (e^{-eV_0过过z_{12}V_e}) = 1
$$

where $\alpha^0 = k_{01}/k_{10}$ and $\beta^0 = k_{12}/k_{21}$. It follows that a plot of $V_0$ vs. In [Na] yields a limiting slope = $kT e(z_{01} + z_{12})$ [mV/e-fold change in [Na]] or $\approx 58 (z_{01} + z_{12})$ mV/10-fold change in [Na] at 20°C. It is important to note that, if $z_0 + z_1 = n$, the limiting slope will be independent of n (58 mV/10-fold [Na]).
where $\alpha = k_{01}/k_{10}$, $\beta = k_{12}/k_{21}$, $\gamma = k_{23}/k_{32}$, $\delta = k_{12}/k_{21}$, and $\epsilon = k_{23}/k_{32}$.

To examine the properties of this model, we make the simplifying assumption that the net charge is translocated across the transmembrane field, i.e., $z_{01} + z_{12} + z_{23} = z_{01} + z_{12} + z_{23} = 1$. The voltage at which 50% of the total charge has moved ($V_{0.5}$), for finite [Na], is found by setting $Q = 0.5N_a e$ in Eq. A10:

$$Q^V = -N_a e \left[ \frac{\alpha \delta (z_{01} + z_{12}) + \alpha \beta (z_{01} + z_{12}) + \alpha \delta (z_{01} + z_{12}) + \alpha \beta y (z_{01} + z_{12} + z_{23}) + \alpha \delta (z_{01} + z_{12} + z_{23})}{1 + \alpha + \alpha \beta + \delta + \alpha \beta y + \alpha \delta} \right]$$

(A10)

Equation A11 can then be solved numerically for $V_{0.5}$.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Eva Hänsenberger for oocyte preparation.

**GRANTS**

This work was supported by grants from the Swiss National Science Foundation (to I.C. Forster) and the Benzoni, Olga Mayenfisch, and Theodor and Ida Herzog-Egli Foundations (to A.-K. Meinild).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**